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Effect of ABT on the Activity and Rate of Degradation of Isoproturon in Susceptible and Resistant Biotypes of *Phalaris minor* and in Wheat

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Abstract: The effect of the monooxygenase inhibitor, 1-aminobenzotriazole (ABT) on isoproturon phytotoxicity and metabolism was studied in resistant (R) and susceptible (S) biotypes of *Phalaris minor* and in wheat (*Triticum aestivum*). Addition of ABT (2·5, 5 and 10 mg litre^{-1}) to isoproturon (0·25, 0·5, 1, 2 and 4 mg litre^{-1}) in the nutrient solution significantly enhanced the phytotoxicity of isoproturon against the R biotype. Isoproturon at 0·25 mg litre^{-1} reduced the dry weight (DW) of the S biotype by 77%, whereas the R biotype required 4·0 mg litre^{-1} for similar reduction. Addition of 10 mg litre^{-1} of ABT to the 0·25 mg litre^{-1} isoproturon caused 71 and 82% reduction in DW of R and S biotypes, respectively. Wheat was more sensitive to the mixture of isoproturon and ABT than the R biotype of *P. minor*. Reduced concentrations of ABT in the mixture from 10 to 2·5 mg litre^{-1} increased the DW of the R biotype more than that of the S biotype.

The R biotype metabolised [^{14}C]isoproturon at a faster rate than the S biotype. ABT (5 mg litre^{-1}) inhibited the degradation of [^{14}C]isoproturon in both biotypes of *P. minor* and in wheat. In the presence of ABT, about half of the applied [^{14}C]isoproturon remained as parent herbicide in all the three species after two days. The metabolites were similar in the R and S biotypes and wheat as determined by co-chromatography with reference standards and mass spectroscopy (MS). ABT inhibited the appearance of the hydroxy and monomethyl metabolites and their conjugates in all the test plants.

These results suggest that the activity of the enzymes responsible for the degradation of isoproturon is greater in the R than in the S biotype of *P. minor*, resulting in its rapid detoxification. Incorporation of the monooxygenase inhibitor ABT into the nutrient solution greatly inhibited the degradation of [^{14}C]isoproturon in the R biotype and increased its phytotoxicity. Both hydroxylation and *N*-dealkylation reactions were found to be sensitive to ABT; inhibition of hydroxylation was greater than that of demethylation. Since ABT could not completely suppress isoproturon degradation, it is possible that more than one monooxygenase is involved. © 1998 SCI

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1 INTRODUCTION

Herbicide-resistant weed populations have evolved in response to the selection pressure imposed by herbicides targeting specific physiological or biochemical processes. Herbicide resistance has occurred under both high (monogenic) and lower (polygenic) use rates.[1] Factors such as initial gene frequency, gene flow, inheritance, seed longevity and fitness of the weed influence evolution of resistance, but not to the same extent as selection pressure.[2] Since the discovery of triazine resistance, there have been numerous reports of weed biotypes exhibiting resistance to photosystem II (PS II)-inhibiting herbicides[3,4] and to date more than 209 weed species have evolved resistance to herbicides of different chemistries (Reference 5 and subsequent data). Resistance to triazines evolved due to high selection pressure caused by their persistent nature and higher and more frequent rates of application than other herbicides. In the case of *Phalaris minor* Retz. (littleseed canarygrass), however, continuous use of lower-than-recommended herbicide rates for 10–15 years has resulted in the evolution of resistance to isoproturon in India.[1,6] The evolution of resistant (R) biotypes was triggered by application of isoproturon as a broadcast mixture with sand and urea fertiliser combined with lower-than-recommended dose rates and a repetitive cropping pattern (rice–wheat rotations).[7] Resistance first documented in 1991–92 in Haryana State has now increased in intensity and magnitude in many areas in Haryana and the adjoining states, particularly Punjab. The control of the R biotypes required an 8–10 times higher dose of isoproturon for the same level of control as that of the susceptible (S) biotypes, and wheat is killed when these rates are used.[8,9]

In general, there could be three potential mechanisms which might confer resistance in *P. minor* to isoproturon. First, reduced target-site delivery due to reduced uptake/translocation or sequestration; second, increased breakdown of the herbicide by a significant increase in the metabolising enzyme system; or third, alteration in the binding site in the D1 protein of PS II. Previous work in our laboratory has shown that target-site alterations were not observed in the Indian R biotypes of *P. minor* as in-vitro oxygen evolution (photosynthesis) was equally inhibited in R and S biotypes by isoproturon; recovery, however, was greater in the R biotype under in-vivo conditions.[10] Similarly, chlorophyll fluorescence was significantly inhibited when the leaves of either R or S biotypes were incubated in isoproturon solution for 4 h. The R biotype recovered completely within 24 h when the leaves were removed from herbicide solution, whereas the S biotype could not recover.[10] Target-site resistance (ACCase) to fenoxaprop-P has been reported in a biotype of *P. minor* from Israel, but this biotype has no resistance to isoproturon or a similar phenylurea herbicide, methabenzthiazuron.[11] No significant differences were observed in the uptake and translocation of [^{14}C]isoproturon in the R (KR-1) and S (H-2) biotypes of *P. minor* from India, though preliminary studies showed that degradation was greater in the R biotype.[9] Wheat degrades isoproturon and chlorotoluron (a phenylurea herbicide like isoproturon) by ring-alkyl hydroxylation and *N*-demethylation due to the action of cytochrome P-450 monooxygenase enzymes[12,13] the first being the major degrading pathway in chlorotoluron.[13] The major metabolites of chlorotoluron in the susceptible weeds, *Avena fatua* L., *Alopecurus myosuroides* Huds. and *Lolium perenne* L. were of *N*-demethylation.[14] The mixed function oxidase inhibitor, ABT (1-aminobenzotriazole), has been found to inhibit the enzymes responsible for the degradation of isoproturon and chlorotoluron in wheat.[12,14] Ring-methyl hydroxylation of chlorotoluron was strongly inhibited by ABT in wheat, but *N*-dealkylation was found to be less affected.[14] The objective of the present study was to investigate the mechanisms of isoproturon resistance by studying the effect of ABT on isoproturon activity and degradation of [^{14}C]isoproturon using the R and S biotypes of *P. minor* and wheat.

2 MATERIALS AND METHODS

2.1 Plant material

Seeds of the putatively R biotype of *P. minor* (KR-1) were collected from a farmer's field from Kurukshetra District of Haryana State, India during 1991–92. The field had a history of continuous use of isoproturon (a single application each year) since the late 1970s, having been under a rice–wheat rotation system of wheat cultivation; in recent years (post-1990) weed control was poor even with increased doses of isoproturon.[6] The S biotype (H-2) was collected during 1990–91 from the research fields of Haryana Agricultural University, Hisar, India where over the years crops and herbicides had been rotated. The most widely cultivated variety of wheat (cv. WH-147) was collected from a farmer's field during 1993–94.

2.2 Activity study with ABT

Plants were grown in a glasshouse with conditions set for 22°/15°($\pm$3)°C day/night temperature and a photoperiod of 14 h supplemented by mercury vapour lamps at 198 mE m^{-2} s^{-1} photon flux density. A sandy loam texture soil was mixed with coarse grit (4 mm size) in 2 : 1 ratio and slow-release fertiliser ('Osmocote'; 15 + 10 + 12, N, P, K) added at 2·5 g kg^{-1} soil. Optipots (9-cm) were seeded with 20–25 seeds of R (KR-1)

and S (H-2) biotypes of *P. minor* and 12–15 seeds per pot of wheat. Plants were thinned after germination and 10 plants per pot were maintained. After three weeks the plants were removed from soil and the roots washed. Plants of uniform growth and vigour were selected and inserted into 50-ml bottles containing liquid nutrient solution (0·75 mM $Ca(NO_3)_2$, 2·5 mM KNO_3, 0·5 mM KH_2PO_4, 0·75 mM $MgSO_4$, 1 mM $NaNO_3$, 9·22 μM Ferric EDTA, 9·22 μM H_3BO_3, 0·16 μM $CuSO_4$, 14·1 μM KCl, 3·6 μM $MnSO_4$, 0·106 μM NH_4 MO_7 O_{24}, 0·77 μM $ZnSO_4$). The plants were acclimatised for three days in a growth room maintained at 29°/20°(±1)°C day/night temperature, 83/56% relative humidity (RH) and a photoperiod of 16 h provided by fluorescent lamps (83 μE m^{-2} s^{-1} photon flux density). After three days the nutrient solution was replaced with fresh nutrient solution containing ABT (Sigma) (0, 0·625, 1·25, 2·5, 5·0, 10·0 mg $litre^{-1}$), isoproturon (Sabre 553 g $litre^{-1}$ SC, AgrEvo; 0, 0·25, 0·50, 1·0, 2·0, 4·0 mg AI $litre^{-1}$) or combinations of isoproturon (0, 0·25, 0·50, 1·0, 2·0, 4·0 mg $litre^{-1}$) + ABT (2·5; 5·0 and 10·0 mg $litre^{-1}$). Three replicate plants of the R and S biotypes of *P. minor* and wheat were used for each treatment and the herbicide/nutrient solution was replenished as required. Observations were recorded periodically on visible mortality, nutrient solution requirement (±ABT/herbicide) and fresh/dry weight (FW/DW) at harvest. Treated plants were harvested after two weeks. The experiment was repeated twice under similar conditions and results from one experiment are presented as representative.

2.3 [^{14}C]isoproturon metabolic studies

For the metabolic studies, the roots of plants grown in the glasshouse in sandy loam soil, as previously described, were washed and plants transferred to a half-strength Hoagland nutrient solution in 50-ml bottles, with three replicate plants per species, and placed in a growth room (as above). Two days before treating with [^{14}C]isoproturon, half of the plants (36) were transferred to nutrient solution with 5 mg $litre^{-1}$ ABT. Before treatment with [^{14}C]isoproturon all plants were sprayed with unlabelled isoproturon (0·25 kg AI ha^{-1}, GR_{50} dose for the S biotype) using a motorised track sprayer fitted with a flat fan even-spray nozzle delivering 400 litre ha^{-1} at 270 kPa. At the three-to-four-leaf stage 0·054 μCi radioactivity per plant was applied on the midrib of the second-oldest leaf as 5 × 0·5 μl drops, using a Hamilton microsyringe. To increase the activity of isoproturon in the label, unlabelled isoproturon (Sabre 553 g $litre^{-1}$ SC; 0·25 kg AI ha^{-1}) was added to [*phenyl-U*-^{14}C]isoproturon (11·97 MBq mg^{-1}; 99% pure) to give a final concentration of 1130 μg ml^{-1} of isoproturon. Leaves of periodically harvested plants (4, 24 and 48 hours after treatment (HAT) with and without ABT) were rinsed with 80% methanol to remove surface residues and stored at −20°C. Even 168 HAT, autoradiography of [^{14}C]isoproturon showed negligible basipetal movement of the label applied to the leaf (data not shown) and hence only treated leaves were harvested for the extraction of metabolites. Frozen leaves were homogenised in 80% methanol and centrifuged at 14 500*g* for 30 min at 2°C. The pellet obtained was washed three times with 80% methanol and the supernatant collected. The combined supernatants were evaporated to dryness using a rotary evaporator, redissolved in 4 ml of 80% methanol and cleaned up by elution through a reverse-phase solid phase extraction cartridge (C-18 Sep Pak). The eluants were dried in a sample concentrator (Savant, Speed Vac Plus SC210A) at 43°C and re-dissolved in 250 μl of 80% methanol. Thin layer chromatography (TLC) plates (Sil 60G-25/UV_{254} and LK6DF of 0·25 mm thickness) were spotted with 20-μl aliquots and eluted with acetone + hexane (1 + 1 by volume). The plates were air dried and [^{14}C]-labelled components detected and quantified using a 'Fujix BAS 1000' Phosphor Image System with TINA (Raytest) evaluation software. The peaks were identified by co-migration with reference standard metabolites (isoproturon, monomethyl-, didesmethyl-, hydroxy-isoproturon, carboxylic acid and isopropyl aniline) (kindly gifted by Rhône-Poulenc Agriculture Limited, UK). The experiment was repeated and the metabolites formed were combined after phosphor image analysis for mass spectroscopic (MS) analysis to identify the metabolites. Analysis was carried out on a VG Quattro triple quadrupole mass spectrometer fitted with a megaflow electrospray source (positive ion electrospray) at Rhône-Poulenc Agriculture Limited, UK. Aliquots were concentrated (>1 μg ml^{-1}) and injected on to a 10-cm Hypercarb S column using acetonitrile + water (60 + 40 by volume) solvent system. The maximum flow rate for MS was 1 ml min^{-1}. The metabolites were identified by comparison with retention times of reference standards and molecular ions. Multiple Reaction Monitoring (MRM) was used for conforming the metabolites identified in full scan MS analysis.

As the metabolites extracted from the three replicated plants were combined to increase the detection precision, no statistics could be applied, hence data for the repeat experiment for rate of degradation of [^{14}C]isoproturon in the three species are also presented.

3 RESULTS

3.1 Activity studies

Based on visible mortality, the R biotype required a much higher dose of isoproturon than the S biotype for

the same level of control (Table 1). The visible mortality in the R biotype was only 30% at 2·0 mg litre^{-1} of isoproturon compared to 75 and 23% in the S biotype and wheat, respectively, at the lowest concentration used (Table 1). There was 100% mortality at 4·0 mg litre^{-1} of isoproturon in both wheat and the R biotype of *P. minor;* the adverse effect of the herbicide was more on wheat than on the R biotype.

Addition of ABT to isoproturon significantly increased its phytotoxicity against the R biotype and wheat (Table 1). The GR_{50} values for the mixture cannot be calculated precisely as mortality was $> 50\%$ at the lowest concentration of isoproturon + ABT treatments. Lowering the concentration of ABT from 10 to 2·5 mg litre^{-1} in the herbicide solution significantly reduced the mortality of all the test plants; the effect, however, was greater in the R than in the S biotype of *P. minor*. There was complete mortality of the R biotype at 0·5 mg litre^{-1} of isoproturon when mixed with 5 or 10 mg litre^{-1} of ABT (Table 1).

The water requirement (WR) was significantly higher in the R than in the S biotype of *P. minor* under treated (isoproturon ± ABT) and untreated conditions (Fig. 1). At 2·0 mg litre^{-1} of isoproturon the WR of wheat and R and S biotypes was 102, 408 and 46 ml for three plants, respectively, for the treatment duration of two weeks (Fig. 1). Addition to ABT (10 mg litre^{-1}) to isoproturon (2·0 mg litre^{-1}) reduced the WR of the above plant species to 41, 35 and 24 ml for three plants, respectively. The R biotype plants treated with 0·25 + 10 mg litre^{-1} of isoproturon and ABT required more than twice the amount of nutrient solution than the S biotype (Fig. 1). The WR of the R biotype was less affected by ABT but in the case of the S biotype and wheat it decreased by 16 and 29%, respectively at 10 mg litre^{-1} concentration compared to the control plants (Fig. 1).

The effect on WR was also reflected in the accumulation of dry weight (DW) by the test plant species (Figs 2–4). Isoproturon at 1·0 mg litre^{-1} reduced the DW of the S biotype by 85% compared to only 3% reduction in the R biotype (Fig. 2). Significant reduction in DW of the R biotype occurred at 2·0 mg litre^{-1}. The DW of the R biotype at 4 mg litre^{-1} was comparable to that of the 0·25 mg litre^{-1} concentration of isoproturon in the S biotype (Fig. 2). The reduction in DW of wheat was greater than in the R biotype at all concentrations of isoproturon. DW was unaffected at lower concentration of ABT in all the three test species, but at 10 mg litre^{-1} it was reduced by 14–18% (Fig. 3). Addition of ABT to isoproturon treatments significantly enhanced the activity of isoproturon against the R biotype (Fig. 4). The DW of the R biotype was reduced by 71% when 10 mg litre^{-1} ABT was added to 0·25 mg litre^{-1} isoproturon solution (Fig. 4) compared to only 5% reduction by isoproturon alone (Fig. 2). The corresponding reduction in the DW of the S biotypes was 82 and 77%, respectively. Reducing the concentration of ABT from 10 to 2·5 mg

TABLE 1
Effect of ABT on the Phytotoxicity of Isoproturon against R and S Biotypes of *Phalaris minor* and Wheat

	Mortality (%)[a]				
	Isoproturon concentration (*mg litre*$^{-1}$)				
Species	*0·25*	*0·5*	*1·0*	*2·0*	*4·0*
Isoproturon alone					
KR-1 (R)	2 (0)	18 (10)	18 (10)	33 (30)	88 (100)
H-2 (S)	60 (75)	88 (100)	88 (100)	88 (100)	88 (100)
Wheat	29 (23)	44 (48)	48 (55)	61 (77)	88 (100)
Isoproturon + ABT (10 mg litre^{-1})					
KR-1 (R)	61 (77)	88 (100)	88 (100)	88 (100)	88 (100)
H-2 (S)	88 (100)	88 (100)	88 (100)	88 (100)	88 (100)
Wheat	51 (60)	88 (100)	88 (100)	88 (100)	88 (100)
Isoproturon + ABT (5·0 mg litre^{-1})					
KR-1 (R)	58 (72)	88 (100)	88 (100)	88 (100)	88 (100)
H-2 (S)	72 (90)	88 (100)	88 (100)	88 (100)	88 (100)
Wheat	45 (50)	67 (85)	88 (100)	88 (100)	88 (100)
Isoproturon + ABT (2·5 mg litre^{-1})					
KR-1 (R)	44 (50)	69 (87)	88 (100)	88 (100)	88 (100)
H-2 (S)	69 (87)	88 (100)	88 (100)	88 (100)	88 (100)
Wheat	44 (48)	65 (82)	88 (100)	88 (100)	88 (100)
LSD (5%)			2.0		

[a] Arcsin transformed data (original values in parentheses).

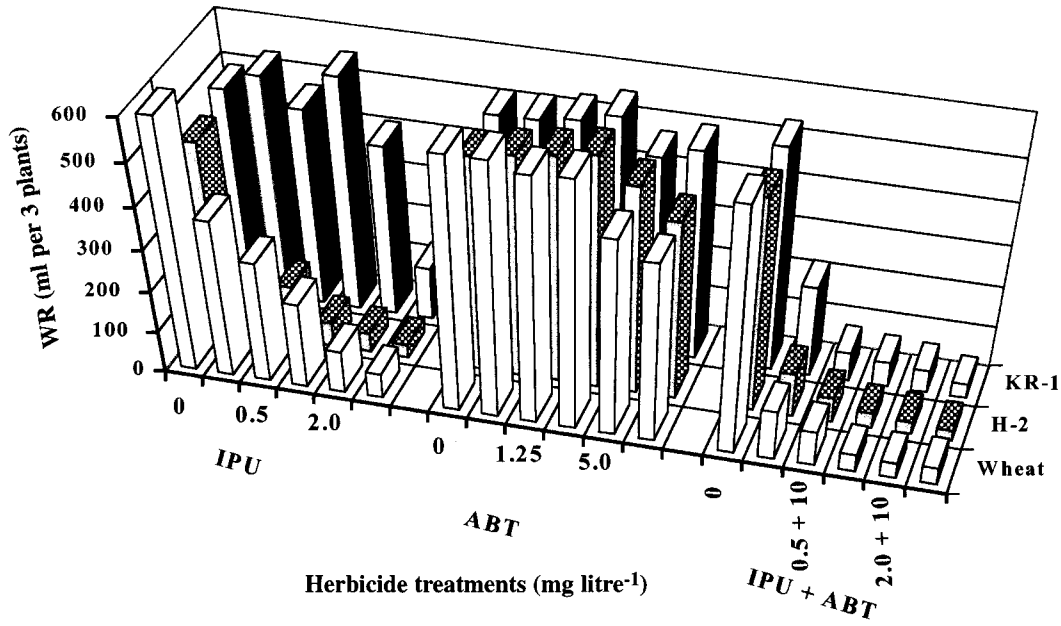

Fig. 1. Water requirement (WR) of wheat and S (H-2) and R (KR-1) biotypes of *Phalaris minor* as affected by isoproturon, ABT and their mixture.

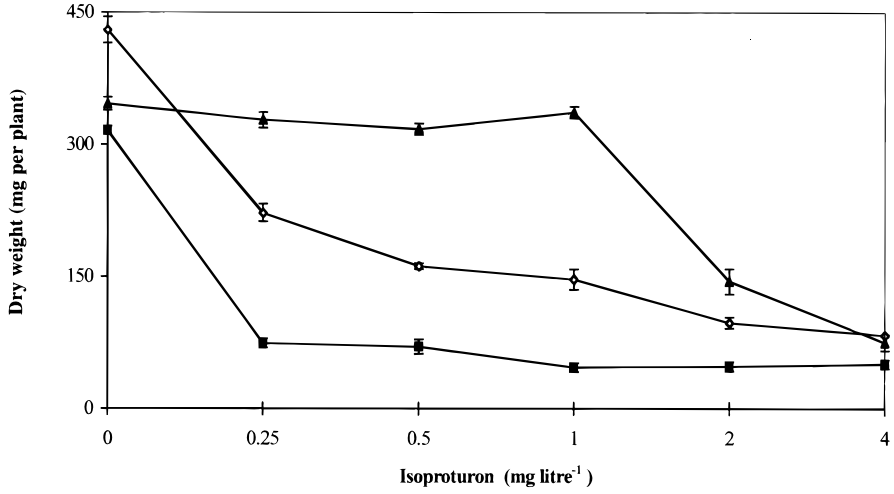

Fig. 2. Effect of isoproturon on dry weight of (◇) wheat and (■) S (H-2) and (▲) R (KR-1) biotypes of *Phalaris minor*. Bar = SD ($n = 3$).

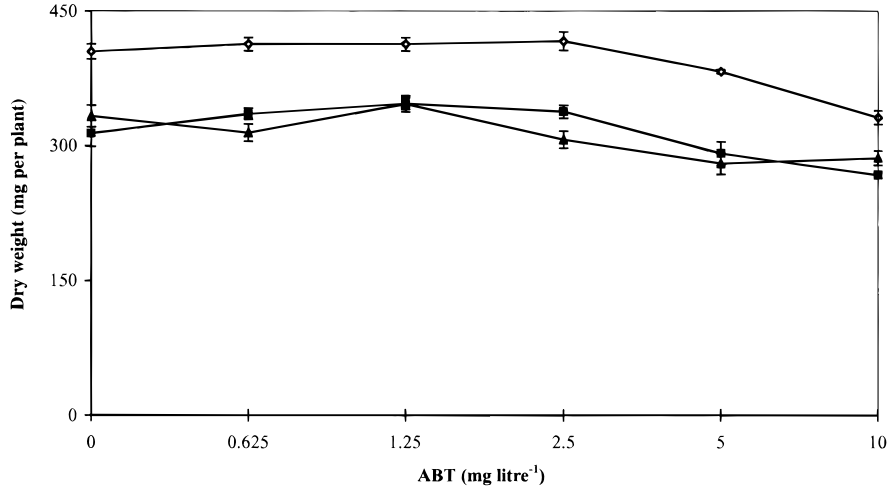

Fig. 3. Effect of ABT on dry weight of (◇) wheat and (■) S (H-2) and (▲) R (KR-1) biotypes of *Phalaris minor*. Bar = SD ($n = 3$).

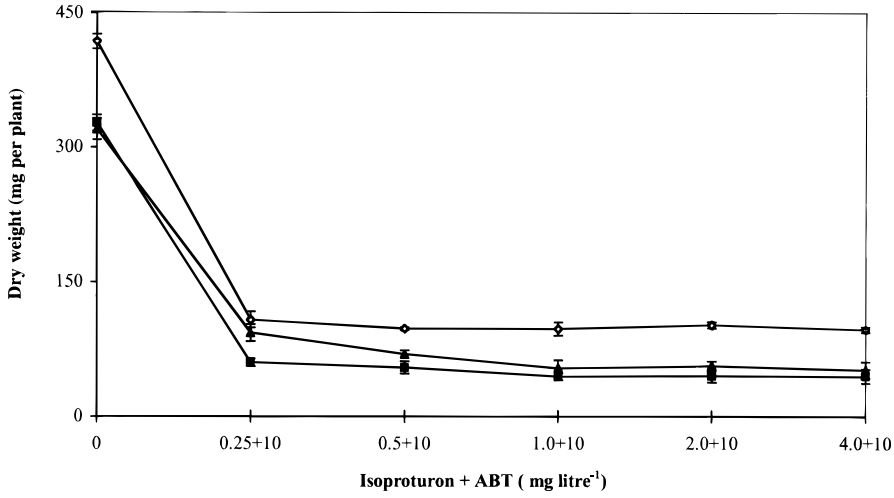

Fig. 4. Synergy of ABT with isoproturon on the dry weight of (◇) wheat and (■) S (H-2) and (▲) R (KR-1) biotypes of *Phalaris minor*. Bar = SD ($n = 3$).

litre^{-1} in an isoproturon concentration of 0·25 mg litre^{-1} increased DW of the R biotype by 31% compared to 20% in the S biotype (data not shown).

3.2 Metabolism studies

In the metabolism study more than 90% of the applied [^{14}C]-label was recovered (surface washes + leaf extracts). TLC analysis of extracted metabolites with phosphor image analysis provided a proportional amount of the metabolites applied on each profile on the basis of 100%. The rate of degradation was found to be faster in the R than in the S biotype of *P. minor* (Table 2 and Fig. 5). The recovery of the [^{14}C]-label from the S biotype was 20, 34, 41 and 55% higher than from the R biotype 4, 24, 48 and 168 HAT, respectively (Table 2). The proportional recovery of [^{14}C]-label was similar from wheat and the R biotype of *P. minor*. Qualitatively similar results were recorded in the repeat experiment, where 42, 66 and 43% of the [^{14}C]-label was found to be unmetabolised 168 HAT in the R and S biotypes of *P. minor* and wheat, respectively (Fig. 5).

Addition of ABT (5 mg litre^{-1}) to the nutrient solution greatly inhibited the degradation of [^{14}C]-label in the R and S biotypes of *P. minor* and wheat (Table 2 and Fig. 5). In ABT-treated plants 48 HAT, the recovery of unmetabolised [^{14}C]-label was 30, 8 and 33% higher in the R and S biotypes of *P. minor* and wheat, respectively, compared to those treated with isoproturon alone (Table 2). In the repeat experiment these differences in the ABT-treated over untreated plants were 23, 11 and 19%, respectively in the R and S biotypes of *P. minor* and wheat 48 HAT and increased to 40, 20 and 38% at 168 HAT (Fig. 5). The proportional recovery of [^{14}C]-label from the S biotype 168 HAT was 57% higher than from the R biotype in the absence of ABT compared to 17% when ABT was added to the nutrient solution (Fig. 5). The rate of degradation of [^{14}C]-label was 32 to 44% higher in the R biotype in the absence of ABT in the nutrient solution under all

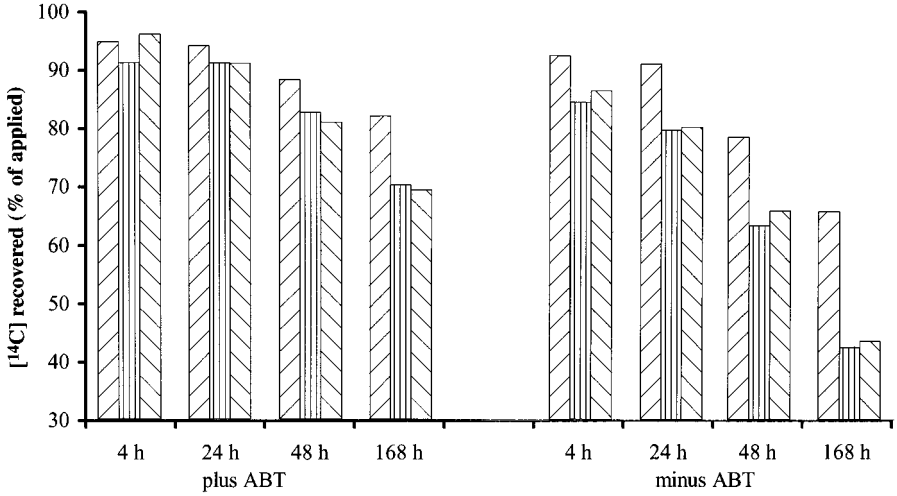

Fig. 5. Recovery of parent isoproturon 4 to 168 HAT as affected by ABT and species. (▨) S (H-2), (▥) R (KR-1) *Phalaris minor*, (▧) wheat.

TABLE 2
Effect of Treatment Period and ABT on the Degradation of [^{14}C] Isoproturon in S and R Biotypes of *Phalaris minor* and Wheat

	[^{14}C] *recovered* (% *of applied*)		
Duration/metabolites	*H-2 (S)*	*KR-1 (R)*	*Wheat*
4 h + ABT			
[^{14}C]-label	73	70	71
Demethylated isoproturon	5	4	7
Hydroxy isoproturon	8	8	10
Glucosidic conjugates	14	18	12
4 h − ABT			
[^{14}C]-label	65	54	60
Demethylated isoproturon	10	10	9
Hydroxy isoproturon	8	14	13
Glucosidic conjugates	16	22	14
24 h + ABT			
[^{14}C]-label	69	62	68
Demethylated isoproturon	7	8	10
Hydroxy isoproturon	9	11	12
Glucosidic conjugates	16	19	10
24 h − ABT			
[^{14}C]-label	59	44	53
Demethylated isoproturon	11	13	11
Hydroxy isoproturon	10	14	12
Glucosidic conjugates	21	28	23
48 h + ABT			
[^{14}C]-label	60	56	54
Demethylated isoproturon	12	9	9
Hydroxy isoproturon	11	12	14
Glucosidic conjugates	15	23	23
48 h − ABT			
[^{14}C]-label	55	39	36
Demethylated isoproturon	11	14	10
Hydroxy isoproturon	12	15	14
Glucosidic conjugates	22	33	39
168 h + ABT			
[^{14}C]-label	57	41	43
Demethylated isoproturon	13	11	10
Hydroxy isoproturon	10	15	18
Glucosidic conjugates	20	34	29
168 h − ABT			
[^{14}C]-label	48	31	35
Demethylated isoproturon	9	9	14
Hydroxy isoproturon	15	16	11
Glucosidic conjugates	27	45	39

treatment durations compared to 9 to 20% in the S biotype (Table 2).

The metabolites of isoproturon formed were of a similar nature in the R and S biotypes of *P. minor*, but the proportions of individual metabolites varied (Table 2). There was a greater proportion of hydroxy metabolites as well as conjugates in the R than in the S biotype at all treatment durations. In the absence of ABT, the proportions of demethylated and hydroxy metabolites were consistently greater in the R biotype (Table 2). The proportion of conjugated metabolite was 1·5 times more than [^{14}C]-label in the R biotype, 168 HAT. Isopropyl aniline metabolite was also observed, but it did not account for more than 4% of the total, and has not been shown in Table 2. Full scan MS analysis detected [^{14}C]-label isoproturon, monomethylated isoproturon, didesmethylated isoproturon, hydroxy isoproturon, carboxylic acid metabolite and isopropyl aniline in the pooled samples, which were further confirmed by MRM analysis (data not presented).

4 DISCUSSION

The dose of isoproturon required to achieve a 50% reduction in growth (GR_{50}) in the R biotype has been found to be 8–10 times higher than that of the S biotype.[9] These differential whole-plant responses were further exemplified in this study in the nutrient solution between the R and S biotypes and wheat, the R biotype requiring a 16 times higher dose of isoproturon than the S biotype (Table 1). The corresponding dose rates were much higher in the R biotype of *P. minor* than in wheat, thus the ultimate level of isoproturon resistance expressed in the R biotype of *P. minor* is high. The significantly higher WR by the R biotype at all concentrations of isoproturon (Fig. 1) and greater dry weight accumulation in isoproturon-treated plants (Fig. 2) indicates its higher resistance.

No visible mortality was observed with ABT alone in any of the test plant species; however, its higher concentration was found to reduce WR and lead to a reduced DW in all the test species (Figs 1 and 3). The adverse effect of ABT on wheat is contrary to previous reports,[15,16] whereas a reduction in foliage fresh weight by 10 to 30% has been reported in *A. myosuroides*.[17] The mixture of ABT and isoproturon was much more effective in reducing the DW of the R biotype of *P. minor* than isoproturon alone. The mixture was also more phytotoxic in wheat, as evidenced by the greater reduction in the DW and also reported earlier.[15,16] The effect of ABT in reducing the DW of the S biotype was less than with the R biotype of *P. minor*, as isoproturon itself caused greater phytotoxicity to the former. Similar results with ABT and isoproturon in the S and R biotypes of *A. myosuriodes* have been observed.[17,18] Resistance to isoproturon in the R biotype of *A. myosuroides* was reported to be due to increased herbicide degradation by monooxygenase enzymes.[18]

Decreasing the concentration of ABT in the nutrient solution from 10·0 to 2·5 mg litre^{-1} also significantly decreased the effect of isoproturon on the R biotype of *P. minor* (Table 1). Such variations were not apparent in *A. myosuroides* with isoproturon mixed with 5·0 or 10·0 mg litre^{-1} of ABT.[17] This could be due to increased activity or levels of monooxygenases in the R compared to the S biotype of *P. minor*, resulting in less binding and oxidation at the lower dose of ABT. There is evidence that ABT is oxidised to benzyne, which

covalently binds to two vicinal nitrogens of the prosthetic haem and thus irreversibly inactivates the enzymes.[18]

The metabolic processes of *N*-dealkylation, *N*-dealkoxylation, ring-alkyl oxidation, ring methyl hydroxylation and some analogous reactions are enzymatically controlled by cytochrome P-450 monooxygenases.[18] These, as well as other enzymes with a haem group at their active centre, are damaged by suicide substrates that bind to the haem group and make them unavailable for oxidation. ABT is one of these suicide substrates which inhibits degradation of isoproturon and works synergistically to make the resistant plant susceptible.[12,15–18] The R biotype of *P. minor* has been found to degrade [^{14}C]isoproturon at a faster rate than the S biotype and wheat.[9] In the present experiment 46% of the applied [^{14}C]isoproturon was metabolised 4 HAT in the R biotype compared to 35 and 40% in the S biotype and wheat, respectively, when ABT was not present in the nutrient solution (Table 2). The R biotype of *P. minor* and wheat converted 58 and 57% of the applied [^{14}C]isoproturon to metabolites 168 HAT, compared to 34% in the S biotype (Fig. 5). Incorporation of ABT in the nutrient solution severely suppressed the degradation of isoproturon in the R biotype of *P. minor* and wheat; the effect, however, was less in the case of the S biotype (Table 2 and Fig. 5). ABT inhibits the metabolism of chlorotoluron and isoproturon in wheat.[12] These herbicides were putatively degraded by the enzymatic action of ring-alkyl hydroxylation of chlorotoluron and of isoproturon and the second *N*-demethylation of isoproturon.[12]

The metabolic pathway of chlorotoluron has been found to be different in the tolerant crop and S and R biotypes of weeds. Wheat was reported to detoxify chlorotoluron primarily by hydroxylation of the ring methyl; *Veronica persica* Poir. degraded chlorotoluron by *N*-dealkylation whereas both pathways were involved in *Bromus sterilis* L. and *Galium aparine* L.[14] The monodemethylated and ring-hydroxylated metabolites of chlorotoluron were found in similar amounts in the S biotype of *A. myosuroides;*[19] in the R biotype, however, aryl ring-hydroxylated metabolite was found in higher proportions than in the S biotype.[20] In the present study, the major metabolites of isoproturon formed by wheat and *P. minor* biotypes were similar in nature but different in amounts. The hydroxy-isoproturon metabolite was in higher proportions in the R than in the S biotype under all treatment durations (Table 2). The differences in the formation of demethylated metabolite were less between R and S biotypes compared to hydroxy metabolite. The R biotype of *P. minor* mimics wheat in degrading isoproturon with similar recovery of [^{14}C]-label and formation of metabolites.

Two primary reactions of isoproturon degradation have been identified in the R biotype of *P. minor;* putative ring-alkyl hydroxylation and *N*-demethylation, which are similar to those in wheat. The pooled metabolites of different treatment durations for wheat and *P. minor* biotypes subjected to mass spectroscopy (MS) revealed the same metabolites in wheat and *P. minor* biotypes (scan not presented). Isoproturon, isopropylaniline and hydroxy metabolites which appeared in the full scan MS analysis were confirmed by multiple reaction monitoring (MRM). Similarly the monomethyl and carboxylic acid metabolites were detected in full scan MS.

ABT was found to inhibit both hydroxylated and demethylated metabolites of isoproturon; the inhibition, however, was greater on hydroxy than demethylated metabolites (Table 2). Similar results of studies with ABT on ring-methyl hydroxylation and *N*-demethylation of chlorotoluron have been reported.[14,20] Both isoproturon and chlorotoluron are reported to be degraded in wheat by hydroxylation and *N*-demethylation mediated by cytochrome P-450 enzymes.[12,18] In wheat, the *N*-demethylation reaction of chlorotoluron was found to be less sensitive to ABT;[14,21] indirect evidence indicates that each reaction is catalysed by different P-450s and it was suggested that there could be two distinct degrading mechanisms for chlorotoluron.[14]

The proportion of demethylated metabolite was more in the ABT-treated plants of S than R biotypes 48 and 168 HAT (Table 2). *N*-monodemethylation reduces the toxicity of isoproturon and the second demethylation makes it non-phytotoxic. After the removal of both *N*-methyl groups further degradation takes place to provide glucosidic conjugates. Higher proportions of conjugated metabolites were recorded in the absence of ABT in wheat and *P. minor* biotypes (Table 2). The exact nature of these conjugated metabolites was not investigated but ABT inhibited their formation. Inhibition of both chlorotoluron and isoproturon metabolism in wheat by ABT by reducing the formation of their intermediate metabolites and conjugates has been reported.[12]

There was no difference in the uptake of [^{14}C]isoproturon in the R and S biotypes of *P. minor*. Uptake was less in wheat than in the *P. minor* biotypes, whereas translocation has been found to be similar in wheat, S and R biotypes.[9] Similarly, uptake and translocation of [^{14}C]chlorotoluron were not different in the R and S biotypes of *A. myosuroides*,[20] and ABT was reported to have no effect on the uptake and translocation of chlorotoluron or isoproturon in wheat.[12,18] *In-vivo* photosynthesis has been shown to recover rapidly in the R biotype of *P. minor* compared to wheat and no recovery was observed in the S biotype.[10] Similarly, remarkable recovery of the chlorophyll fluorescence in the R biotype after removal from isoproturon solution indicates faster degradation of isoproturon in the R biotype of *P. minor*.[10]

Differences in the rate of degradation in the R biotype of *P. minor*, however, were not as high as with *A. myosuroides* with chlorotoluron and isoproturon.[18,20] The R biotype of *A. myosuroides* metabolised chlorotoluron more than twice as fast as the S biotype. ABT-induced degradation inhibition of these herbicides was also greater in *A. myosuroides*[18,20] than in *P. minor* biotypes. Both species have shown cross-resistance to diclofop-methyl,[22,23] suggesting enhanced degradation mechanisms as a mode of action. The rate of degradation of, and level of resistance to, isoproturon in *A. myosuroides* were found to be lower than those of chlorotoluron,[18] possibly due to greater oxidative breakdown of the methyl group of chlorotoluron.[24] Both herbicides possess similar modes of action and ABT was found to inhibit their degradation in wheat and to increase phytotoxicity when mixed with P-450 inhibitors (ABT and PBO).[12,15,16]

The R biotype of *P. minor* required a much higher dose of isoproturon than the S biotype and wheat to give a similar phytotoxicity level; a rapid recovery of chlorophyll fluorescence and in-vivo photosynthesis suggests that it has acquired a higher level of degrading enzymes. The similar effects of ABT on isoproturon toxicity suggest that the enzymes involved in the breakdown of isoproturon are similar, if not identical in wheat and *P. minor* biotypes. Lowering the concentration of ABT in isoproturon solution resulted in reduced mortality of the R biotype (Table 1), suggesting that it has an elevated level of monooxygenase enzymes. The microsomal protein concentrations of the R and S biotypes were found to be similar (data not presented), from which it could be inferred that the R biotype has an enhanced activity of the enzymes. Contrary to general belief, chlorotoluron provided an excellent control of both R and S biotypes of *P. minor* and no differences were recorded in the R and S biotypes under pot and nutrient culture studies (Singh *et al.*, in preparation). The lower inhibition of [^{14}C]isoproturon degradation by ABT in *P. minor* biotypes and their complete loss of resistance to chlorotoluron suggest that more than one monooxygenase enzyme is involved in the degradation.

It can be conjectured from the above data that the resistance in the R biotype of *P. minor* is due to increased activity of monooxygenase enzymes. The 1·6-times higher rate of [^{14}C]isoproturon degradation in the R biotype suggests that it has evolved metabolic resistance to isoproturon, as no target-site alterations were observed and P-450 inhibitors synergised the activity of isoproturon.

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